STANDARD OPERATING PROCEDURE

Title: Preparation of Soluble Lysates from Tumor Tissue

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Purpose

The purpose of this document is to describe the preparation of a soluble lysate from cryopulverized breast cancer xenograft tissue in a non-denaturing lysis buffer.

Scope

This procedure is used to prepare a pooled tumor lysate from cryopulverized tissue that is used as a test matrix for the development of PRM assays for peptides quantification in tumor digests.

Responsibilities

It is the responsibility of person(s) performing this procedure to be familiar with laboratory safety procedures. The interpretation of results must be done by a person trained in the procedure and familiar with such interpretation.

Equipment

- Covaris S220X Focused Acoustics System with cooling unit
- Micro-centrifuge (Eppendorf, Model No. 5424)
- Rainin™ Pipet-lite XLS, P20, P200, P1000
- Axygen® MAXYmum™ recovery tips: P200 and P20: T-200-C-L-STK, P1000: T-1000-C-L-R.

Materials

Item	Vendor	Catalog #	Unit
12 x12 glass tubes and caps	Covaris	520080	100 per pack
1.7mL Eppendorf tubes	Axygen	MCCT-175-C	
Ultrafree-MC-HV Centrifugal Filters	Millipore	UFC30HVNB	250/box
Dry ice			
Ice			
Pipette tips	Axygen	T-1000-C-L-R	

Reagents

Item	Vendor	Catalog #	Unit
EDTA	Sigma	E1644	100g
EGTA	Sigma	E4378	25g
1M HEPES	Sigma	3537	100mL
Hydrochloric acid	Fisher	A144S-500	500 mL
Phenyl methylsufonyl fluoride	Thermo Scientific	36978	5g
Protease Inhibitor Cocktail-Complete	Roche	11697498001	20 Tablets
Phosphatase Inhibitor Cocktail 2	Sigma	P5726	
Phosphatase Inhibitor Cocktail 3	Sigma	P0044	
Sodium Chloride	Fisher	S271	500g
Sodium Orthovanadate	Sigma	S6508	50g
Sodium Fluoride	Sigma	S7920	100g
Triton X-100	Sigma	93443	100mL
18.2 mΩ water	Millipore		
Albumin Standard	Pierce	23209	
Advanced Protein Assay Reagent	Cytoskeleton Inc.	ADV01	

Solutions

PMSF (100mM).

a. Weigh 17.4mg of PMSF into a 1.7mL Eppendorf tube.
b. Solubilize in 1 mL of 100% ethanol.
c. Aliquot and store at -20°C.

NaCl (5M)

a. Weigh 29.2 g NaCl into 100mL graduated cylinder half filled with 18.2 mΩ water.
b. Stir to dissolve and Q.S. to 100mL mark.
c. Store at RT.

EDTA (100 mM)

a. Weigh 3.722 g EDTA into 100mL graduated cylinder half filled with 18.2 mΩ water.
b. Stir to dissolve and Q.S. to 100mL mark.
c. Store at RT.

EGTA (250 mM)

a. Weigh 9.509 g EGTA into 100mL graduated cylinder half filled with 18.2 mΩ water.
b. Stir to dissolve and Q.S. to 100mL mark.
c. Store at RT.

HCL (1M)

a. Slowly add 4.106 mL of concentrated HCL solution to 12.5 mL deionized water.
b. Adjust the final volume of solution to 50 mL with deionized water.
c. Store at RT.

NaF (100 mM)

a. Weigh 0.2099g NaF into 50mL graduated cylinder half filled with 18.2 mΩ water.
b. Stir to dissolve and Q.S. to 50mL mark.
c. Aliquot and store at -20C

Na_3VO_4 (100 mM)

a. Weigh .9195g Na_3VO_4 into 50mL graduated cylinder half filled with 18.2 mΩ water.
b. Stir to dissolve and Q.S. to 50mL mark.
c. Adjust pH to 10 using 1 M NaOH or 1 M HCl.
d. Boil solution by heating in a microwave for 5 - 15 seconds. After boiling for 5 - 15 seconds, the solution will be clear and colorless.
e. Cool on ice until the Na3VO4 solution reaches room temperature.
f. Adjust pH to 10 again using of 1 M HCl
g. Repeat steps 4-6 a total of 3-5 times until the pH stablizes at ~10. At this point, adding HCl should result in little, if any, appearance of yellow color in the solution.
h. Aliquot and store activated Na_3VO_4 at -20°C.

Multi-Inhibitor Binding (MIB) Lysis Buffer (Prepare fresh prior to use)

Final Concentration	Stock Sol.	50 mL
50 mM HEPES	1 M	2.5 mL
150 mM NaCl	5 M	1.5 mL
0.5% TritonX-100	10%	2.5 mL
1 mM EDTA	100 mM	0.5 mL
1 mM EGTA	250 mM	0.2 mL
10 mM NaF	100 mM	5 mL
2.5 mM Na_3VO_4	100 mM	1.25 mL
Protease Inhibitor Cocktail (Roche)		2 tablets
Phosphate Inhibitor Cocktail 2	100x	500 µL
Phosphate Inhibitor Cocktail 3	100x	500 µL

Procedure

1. **Set up Covaris S220X**
    a. Set up Covaris S220X. Add approximately 1.55L of DI water to the reservoir. Place reservoir with water under the transducer assembly and push transducer assembly down into the reservoir.
    b. Check water level with 12 x 12 glass tube; the entire glass tube up to the cap should be immersed in water.
    c. Switch on the S220X unit.
    d. Start Sonolab 7.0 software on the laptop. The degas pump will start automatically.
    e. Start the water chiller and set temperature to 4^{O}C.
    f. The Sonolab software will show green check marks when the instrument is ready and the operating temperature has been reached. It will take approximately 30 minutes.

2. **Protein Extraction From Tissue Sample**
    a. Collect tissue samples from -80^{O}C freezer and put them on dry ice until buffer is added.
    b. Add 400µL of prepared MIB lysis buffer to frozen tissue in cryovial. Transfer buffer and tissue to 12x12 glass tube with pipette. It helps to cut off a portion of the pipette tip to increase the diameter of the pipet tip to allow the tissue to be aspirated with the solubilization buffer.
    c. Add an additional 400µL MIB lysis buffer to the cryovial and rinse the vial. Transfer buffer and any remaining tissue to the 12x12 glass tube.
    d. Cap the 12x12 glass tube and place into the tube holder for the Covaris S220X.
    e. Start extraction method (see Table 1 for method parameters).
    f. Once extraction run is completed, remove the 12 x 12 glass tube from the S220X and transfer sample into a 1.7mL Eppendorf tube.
    g. Spin sample at 16,000 rcf for 10 minutes.
    h. Remove supernatant and add to Ultrafree-MC-HV Durapore filter.
    i. Centrifuge for 15 min at 14,000g.
    j. Remove supernatant to a new 1.7 mL Eppendorf tube.
    k. Sample is ready for further processing or can be stored at -80^{O}C.

3. **Shut down Covaris S220X**
    a. When all samples are processed, shut down degas pump in the Sonolab software
    b. Lift transducer assembly out of the water bath and empty water from reservoir.

c. Start the degas pump. The pump will pump out any remaining water in the degas coils.
d. Once the degas pump stops, start it one more time.
e. Lift transducer assembly out of the reservoir and remove all water. Dab transducer assembly dry with a kimwipe. Dry the reservoir with a kimwipe.
f. Place reservoir back under transducer assembly and lower transducer assembly.
g. Exit from Sonolab software, and then shut off the S220X unit and the water chiller.

4. Protein Determination

a. Prepare serial dilutions of 2mg/mL BSA standard in the range of 0.007 mg/mL to 2mg/mL BSA in DI water.
b. Dilute samples 1:10 in water; make 50µL.
c. Dilute lysis buffer 1:10 in water; make 1mL
d. Prepare a 1:50 dilution of each sample using the 1:10 diluted samples by pipetting 24ul of 1:10 diluted lysis buffer into a 0.5mL PCR tube and adding 6µL of the 1:10 diluted sample.
e. Prepare a 1:100 dilution of each sample using the 1:10 diluted samples by pipetting 27µL of 1:10 diluted lysis buffer in a 0.5mL PCR tube and adding 3µLof the 1:10 diluted sample.
f. Pipet 10ul of 1:10 diluted lysis buffer into each standard cuvette, then add 10ul of standard. To the blank cuvette, add 10µL of 1:10 diluted lysis buffer and 10 µL of DI water.
g. Pipet 10ul of water into each sample cuvette, then add 10ul of each sample dilution. Samples are assayed in duplicate.
h. Add 1mL of Advanced Protein Assay Reagent, mix and read in Thermo BioMate 3 spectrophotometer at 590nm.
i. Plot the response curve in Excel and use the linear regression line to calculate sample concentration.

Table 1. Covaris Extraction Parameters	
Peak Incident Power	100
Duty Factor	10%
Cycles per burst	500
Temperature	4°C
Time	2 min

Referenced Documents

- Mertins P (2014) “Ischemia in tumors induces early and sustained phosphorylation changes in kinase pathways but does not affect global protein levels. Mol. Cellular Proteomics. 13, 1690-1704.